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β-Adrenoceptors and dexamethasone synergistically stimulate the expression of the angiotensinogen gene in opossum kidney cells

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β-Adrenoceptors and dexamethasone synergistically stimulate the expression of the angiotensinogen gene in opossum kidney cells. We transiently co-transfected opossom kidney (OK) cells with the plasmid containing the cDNA for β_1-adrenoceptor (pBC-β_1AR) or β_2-adrenoceptor (pBC-β_2AR) and a fusion gene with the 5′-flanking region of the angiotensinogen (ANG) gene linked to a bacterial chloramphenicol acetyl transferase (CAT) coding sequence as a reporter, pOCAT (ANG N-1498/+18). Co-transfection of plasmid pBC-β_1AR or pBC-β_2AR alone enhanced the expression of pOCAT (ANG N-1498/+18). The addition of isoproterenol further stimulated the expression of pOCAT (ANG N-1498/+18) when co-transfected with pBC-β_1AR, but not with pBC-β_2AR. Moreover, the addition of a combination of dexamethasone and isoproterenol synergistically stimulated the expression of pOCAT (ANG N-1498/+18) when co-transfected with pBC-β_1AR, but not when co-transfected with pBC-β_2AR. The synergistic effect of dexamethasone and isoproterenol was inhibited by the presence of RU 486 (an antagonist of glucocorticoid) or Rp-cAMP (an inhibitor of cAMP-dependent protein kinase A I and II). To localize the putative cAMP-responsive element (CRE) and glucocorticoid responsive element (GRE) in the ANG gene, we constructed the fusion gene by inserting the DNA fragment, ANG N-806 to N-465 upstream of the thymidine kinase (TK) promoter fused to a CAT gene and introduced them with pBC-β_1AR into OK cells. The addition of dexamethasone or isoproterenol alone stimulated the expression of pTKCAT (ANG N-806/-465). The addition of isoproterenol and dexamethasone synergistically stimulated the transcriptional activity of pTKCAT (N-806/-465). These studies demonstrate that the β_1-adrenoceptor and dexamethasone act synergistically to stimulate the expression of the ANG gene in OK cells via the putative CRE and GREs in the 5′-flanking region of the rat ANG gene. These data should aid in the understanding of the molecular mechanism(s) of the stimulatory effect of catecholamines/glucocorticoid induced expression of the ANG gene in the kidney.

The existence of an intrarenal renin-angiotensin system (RAS) has now been generally accepted [1, 2]. Studies by Seikaly et al [3] and Braam et al [4] have shown that levels of luminal angiotensin II (Ang II) in the rat renal proximal tubule are as high as 10^{-9} M, whereas levels of plasma Ang II are less than 10^{-12} M. The presence of angiotensinogen (ANG) mRNA has also been localized in rat renal proximal tubules by both techniques of *in situ* hybridization [5] and polymerase chain reaction [6]. Furthermore, studies by Wolf and Neilson [7] and Tang et al [8] demonstrated that ANG mRNA is detected in rat and mouse immortalized renal proximal tubular cells, respectively. We [9], as well as Ingelfinger et al [10], have also demonstrated that the ANG mRNA is expressed in opossum kidney (OK) proximal tubular cells. Thus, these studies strongly indicated that renal Ang II is probably derived from the ANG synthesized in the renal proximal tubules.

We have previously reported that thyroid hormone (L-T_3), dexamethasone, 8-Bromo-cAMP (8-Br-cAMP) and forskolin stimulate the expression of the ANG gene in OK cells *in vitro* in a dose-dependent manner [9, 11]. Furthermore, we demonstrated that isoproterenol stimulates the expression of the ANG-GH fusion gene in OK 27 cells [12]. The effect of isoproterenol could be blocked effectively by the presence of propranolol (β-blocker), atenolol (β_1-blocker) and Rp-cAMP (an inhibitor of cAMP-dependent protein kinase A I and II) [12]. ICI 118,551 (β_2-blocker), however, was not effective (approximately 0.01% as effective as atenolol) in blocking the effect of isoproterenol. Our studies confirm the reports of Nakamura and Johns [13] that the administration of atenolol blocked the effect of renal nerve stimulation on the increase of ANG mRNA level in the rat kidney *in vivo*. Hence, our studies [12] and the studies of Nakamura and Johns [13] indicate that the β_1-adrenoreceptor stimulates the expression of renal ANG gene.

More recently, we demonstrated that the addition of 8-Br-cAMP and dexamethasone act synergistically to stimulate the expression of the ANG gene in OK cells [14]. Since both the β_1- and β_2-adrenoceptors are positively coupled to adenylyl cyclase [15–17], we raise the question whether there is a synergistic effect between β-adrenoceptors and dexamethasone on the expression of the ANG gene in OK cells.

In the present study, we investigated the possible synergistic effect of β-adrenoceptors and dexamethasone on the expression of the ANG gene in OK cells. Our studies demonstrated that the addition of isoproterenol stimulates the expression of the ANG-CAT fusion gene, pOCAT (ANG N-1498/+18) when co-transfected with β_1-adrenoceptors cDNA, but not with β_2-adrenoceptor. Furthermore, dexamethasone and β_1-adrenoceptor acted synergistically to stimulate the expression of the pOCAT (ANG N-1498/+18), whereas no apparent synergistic effect was observed with dexamethasone and the β_2-adrenoceptor. The addition of RU 486 (an antagonist of glucocorticoid) or Rp-cAMP could block the synergistic effect of dexamethasone and isoproterenol on the expression of the ANG gene in OK cells. Finally, we have

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localized the putative CRE and GREs in the DNA fragment ANG N-806 to N-465 of the 5′-flanking region of the rat ANG gene.

Methods

Materials

Restriction and modifying enzymes were purchased either from Bethesda Research Laboratories (Gibco-BRL, Burlington, Ontario, Canada), Boehringer-Mannheim (Dorval, Quebec, Canada) or Pharmacia Inc. (Baie d'Urfe, Quebec, Canada).

The expression vector, pOCAT, pRSVCAT and pTKCAT containing the coding sequence for chloramphenicol acetyl transferase (CAT) with or without Rous Sarcoma Virus enhancer/promoter (RSV) or the thymidine kinase promoter (TK) sequence fused to the 5′-end of the CAT coding sequence, respectively, were a gift from Dr. Joel F. Habener (Lab. of Molecular Endocrinology, Massachusetts General Hospital, Boston, MA, USA). The mammalian expression vector, pBC-BC12, containing the cDNA for human β_1-adrenoceptor (pBC-β_1AR) or the β_2-adrenoceptor (pBC-β_2AR) were constructed as described previously [15–17].

Alpha-[^{35}S]dATP (> 1000 Ci/mmol), alpha-[^{32}P]dCTP (800 Ci/mmol), gamma-[^{32}P]ATP (3000 Ci/mmol), D-Threo-[1, 2^{14}C]-chloramphenicol were purchased from Dupont, New England Nuclear (NEN; Boston, MA, USA).

Rp-cAMP (an inhibitor of the cAMP-dependent protein-kinase A I and II [18]) was purchased from Research Biochemicals Inc. (RBI, Natick, MA, USA).

RU 486 (an antagonist of glucocorticoid) was a gift provided by Dr. Alain Bélanger (Laval University, Quebec, Canada).

Thin layer chromatography plates were purchased from Promega-Fisher Inc. (Montreal, Quebec, Canada). Other molecular biology grade reagents were obtained from Sigma Chemicals (St. Louis, MO, USA), Gibco-BRL, Boehringer-Mannheim, Pharmacia Inc. or Promega-Fisher Inc.

Construction of fusion genes

The methods of construction of pOCAT (ANG N-1498/+18) fusion gene have been described previously [11]. The plasmid pTKCAT (ANG N-806/-465) was constructed by inserting the DNA fragment, ANG N-804 to N-465, upstream of the thymidine kinase (TK) promoter fused to a CAT gene of the modified plasmid, pTKCAT [14]. The double-strand DNA fragment, ANG N-806 to N-465, was synthesized by the method of amplification by polymerase-chain reaction (PCR) (Thermal Cycler) according to the manual supplied by Perkin Elmer Cetus Inc. (Forster City, CA, USA). The isolated DNA fragment, ANG N-814 to N+18 [14] was used as templates to amplify the DNA fragment, ANG N-806 to N-465. The nucleotide sequences for the oligonucleotide primers corresponding to nucleotides ANG N-806 to N-779 (5′AAG AGA TTA CTT GAC TTA CTG GAT GCA A 3′; Primer #1), and ANG N-465 to N-485 (5′AGT TGT CTG TTC TGC ACA GGG 3; Primer #2) were used in PCR. The oligonucleotides were synthesized by Biosynthesis Inc. (Lewisville, Tx, USA). The DNA sequence and orientation of the ANG N-806 to N-465 in the modified pTKCAT [14] was confirmed by dideoxy sequencing with SP6 primer (Promega-Fisher Inc.).

Cell culture

The opossum kidney (OK) proximal tubular cell line was obtained from the American Tissue Culture Collection (ATCC) (Rockville, MD, USA). This cell line is derived from the kidney of a female American opossum and retains several properties of proximal tubular epithelial cells in culture [19, 20] and expresses a low level of ANG mRNA [9, 10]. The culture conditions of OK cells have been described previously [9, 11, 14]. After approximately 15 to 20 passages following reception of the cells from ATCC, they were used for the present studies.

DNA transfection

The method of DNA transfection into OK cells has been described previously [9, 11, 14]. A total of 10 to 20 μg of supercoiled DNA was routinely used in the cell transfection. Briefly, cells were incubated in DMEM containing 10% foetal bovine serum (FBS) immediately after transfection. Twenty-four hours after the transfection, the media were replaced with fresh media without FBS. The cells were harvested 24 or 48 hours later and assayed for CAT activity [11].

To study the effect of isoproterenol or dexamethasone on the expression of the fusion gene [pOCAT (ANG N-1498/+18)] co-transfected with pBC-β_1AR or pBC-β_2AR, the media were changed to serum-free DMEM at 24 hours after DNA transfection. Then various concentrations (10^{-13} to 10^{-5} M) of hormones were added. After an overnight incubation, the cells were harvested for CAT assays.

Plasmids pOCAT and pRSVCAT were used as negative and positive controls, respectively.

To normalize the efficiency of transfection of the fusion gene, 2 μg of pTKGH (a vector with the thymidine kinase (TK) enhancer/promoter fused to the 5′-end of the human growth hormone (hGH) gene) was cotransfected with pOCAT (ANG N-1498/+18) (10 μg DNA) with or without pBC-β_1AR or pBC-β_2AR (1 to 5 μg DNA). Briefly, the levels of immunoreactive human growth hormone (IR-hGH) in the media were quantified by the specific radioimmunoassay for hGH. The levels of IR-hGH in the control group [Fig. 1; cells transfected with pOCAT (ANG N-1498/+18) and pTKGH but without the co-transfection with either pBC-β_1AR or pBC-β_2AR] were used as 100% transfection efficiency. The levels of IR-hGH in other groups [cells co-transfected with pOCAT (ANG N-1498/+18), pTKGH and pBC-β_1AR or pBC-β_2AR] were compared with the control group as % of transfection efficiency. Subsequently, the cellular enzymatic CAT activities in groups co-transfected with either pBC-β_1AR or pBC-β_2AR were normalized with the % of transfection efficiency as compared to controls. In comparison with pRSVCAT (that is, pRSVCAT co-transfected with 2 μg of pTKGH), the transfection efficiency of pOCAT (ANG N-1498/+18) ranged from 35% to 55% as compared to pRSVCAT. The inter- and intra-assay coefficient of variation of transfection for pOCAT (ANG N-1498/+18) in OK cells were 25% (N = 10) and 8% (N = 10), respectively.

The radioimmunoassay for hGH was performed according to the method described previously [9].

Chloramphenicol acetyl transferase (CAT) assay

The method for the CAT assay has been described previously [11]. The results of all CAT assays are given as the mean ± SD of triplicate assays.

Statistical analysis

The experiments were performed at least two to three times in triplicate. The data were analyzed by Student's t-test or analysis of

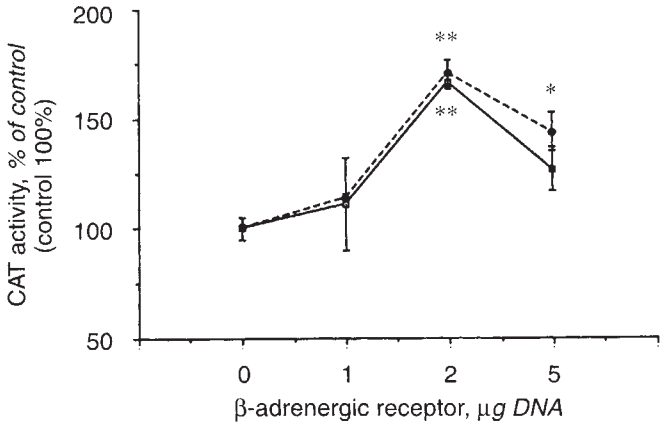

Fig. 1. *Expression of the pOCAT (ANG N-1498/+18) co-transfected with different concentrations of plasmid containing the cDNA for the β_1-adrenergic receptor (pBC-β_1AR) or the β_2-adrenergic receptor (pBC-β_2AR) in OK cells.* Ten μg of pOCAT (ANG N-1498/+18), 2 μg pTKGH and different concentrations (1 to 5 μg) of pBC-β_1AR or pBC-β_2AR per well (5×10^5 cells) were used in these experiments. The levels of transcriptional activity were quantified by CAT-enzymatic activity. The efficiency of transfection was normalized by the amount of IR-hGH in the media. Control cells (without co-transfection with pBC-β_1AR or pBC-β_2AR) were given a relative value of 100% (*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.005$) (⊡, pBC-β_1AR; -●-, pBC-β_2AR).

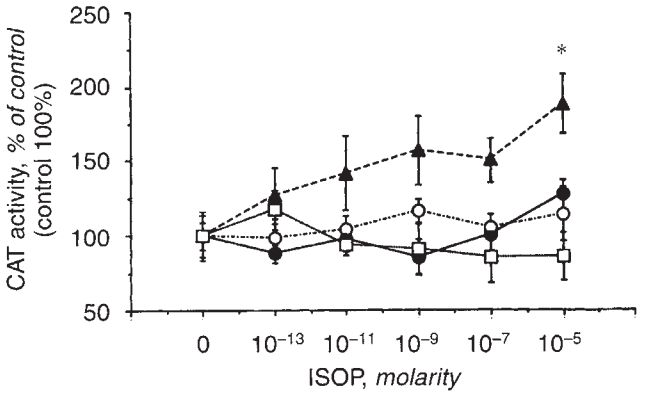

Fig. 2. *Dose-response relationship for the addition of isoproterenol on the expression of pOCAT (ANG N-1498/+18) when co-transfected with pBC-β_1AR or pBC-β_2AR or an empty vector pBC-BC12 in OK cells.* Ten μg of pOCAT (ANG N-1498/+18), 2 μg of pTKGH and 2 μg of BC-β_1AR or pBC-β_2AR or pBC-BC12 per well were used in the experiment. The effect of isoproterenol is compared with the control cells (without addition of isoproterenol, an arbitrary relative value of 100%). The efficiency of transfection was assessed and normalized to the amount of IR-hGH in the media (*$P \leq 0.05$; **$P \leq 0.01$ and ***$P \leq 0.005$) (-▲-, pBC-β_1AR; -●-, pBC-β_2AR; -□-, pBC-BC12; -○-, without co-transfection with pBC-β_1AR or pBC-β_2AR). Similar results were observed from two additional experiments.

variance. A probability level of $P \leq 0.05$ was regarded as significant.

Results

Effect of co-transfection of pBC-β_1AR or pBC-β_2AR on the expression of pOCAT (ANG N-1498/+18) fusion gene in OK cells

Figure 1 shows that co-transfection of pBC-β_1AR or pBC-β_2AR (1 to 5 μg DNA) stimulated the expression of the pOCAT (ANG N-1498/+18). A 1.6- to 1.8-fold stimulation was found with 2 μg of pBC-β_1AR ($P \leq 0.01$). A higher concentration of pBC-β_1AR (that is, 5 μg), did not stimulate the expression of the pOCAT (ANG-N-1498/+18). Similarly, 2 μg of pBC-β_2AR stimulated the expression of pOCAT (N-1498/+18) by 1.6- to 1.8-fold. At 5 μg of pBC-β_2AR, the stimulatory effect of pβ_2-AR was diminished but was still significantly increased by a factor of 1.45-fold ($P \leq 0.05$) over the controls. These studies indicate that the expression of pOCAT (ANG N-1498/+18) is stimulated by either the β_1- or β_2-adrenoceptor alone.

Dose-response curve of isoproterenol or dexamethasone on the expression of pOCAT (ANG N-1498/+18) fusion gene co-transfected with pBC-β_1AR in OK cells

Figure 2 shows that the addition of a high concentration of isoproterenol (10^{-5} M) stimulated the expression of the pOCAT (ANG N-1498/+18) when co-transfected with 2 μg of pBC-β_1AR ($P \leq 0.05$), whereas smaller concentrations (10^{-13} to 10^{-7} M) had no significant effect on the expression of the pOCAT (ANG N-1498/+18). In cells transfected with pOCAT (ANG N-1498/+18) alone or co-transfected with either pBC-β_2AR (2 μg) or pBC-BC12 (2 μg), however, the addition of isoproterenol (10^{-13} to 10^{-5} M) had no significant stimulatory effect on the expression of the pOCAT (ANG N-1498/+18).

Figure 3 shows that the addition of dexamethasone (10^{-12} to

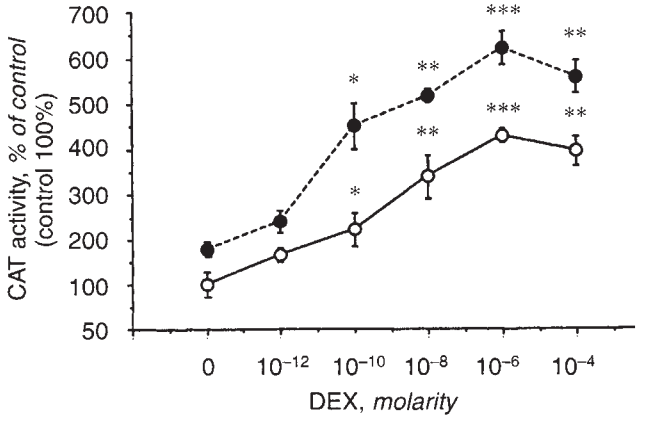

Fig. 3. *Dose-response relationship for the addition of dexamethasone on the expression of pOCAT (ANG N-1498/+18) co-transfected with pBC-β_1AR in OK cells in the presence or absence of isoproterenol (10^{-5} M).* Ten μg pOCAT (ANG N-1498/+18) and 2 μg pBC-β_1AR per well were used in the experiment. The effect of dexamethasone is compared to the control cells (without addition of dexamethasone or isoproterenol, an arbitrary relative value of 100%). Similarly, the effect of a combination of dexamethasone and isoproterenol is compared to the cells in the presence of isoproterenol alone (*$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.005$) (-○-, absence of isoproterenol; -●-, presence of isoproterenol).

10^{-4} M) alone stimulated the expression of the pOCAT (ANG N-1498/+18) when co-transfected with 2 μg of pBC-β_1AR in a dose-dependent manner. The maximal and half-maximal effects of dexamethasone were found with 10^{-6} M ($P \leq 0.005$) and 10^{-10} M ($P \leq 0.05$) dexamethasone, respectively. In the presence of isoproterenol (10^{-5} M), the maximal and half-maximal effects of dexamethasone were significantly higher ($P \leq 0.05$), as compared to those in the absence of isoproterenol.

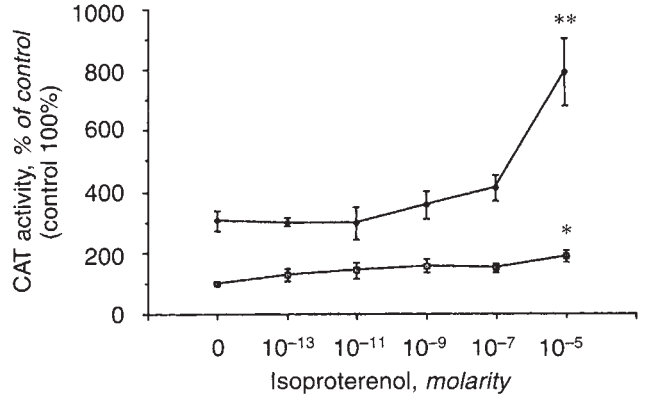

Fig. 4. *Dose-response relationship for the addition of isoproterenol on the expression of pOCAT (ANG N-1498/+18) co-transfected with pBC-β_1AR in OK cells in the presence or absence of dexamethasone.* Ten μg pOCAT (ANG N-1498/+18) and 2 μg pBC-β_1AR per well were used in the experiment. The effect of isoproterenol is compared with the control cells (without addition of dexamethasone and isoproterenol, an arbitrary relative value of 100%). Similarly, the effect of a combination of isoproterenol and dexamethasone is compared to the cells in the presence of dexamethasone (*$P \leq 0.05$; **$P \leq 0.01$ and ***$P \leq 0.005$) (⊡, absence of dexamethasone; -●-, presence of dexamethasone).

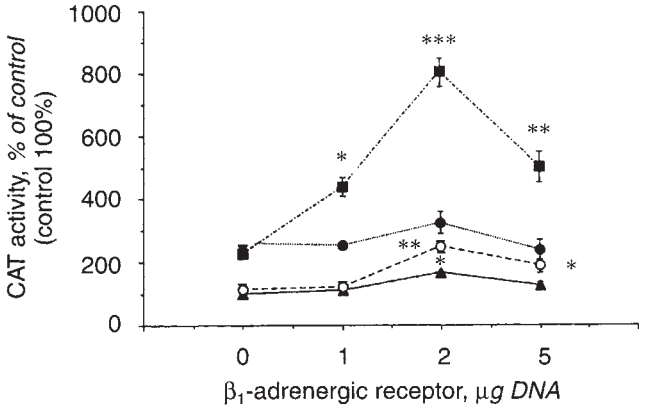

Fig. 5. *Effect of isoproterenol (10^{-5} M) or dexamethasone (10^{-6} M) or a combination of both on the expression of pOCAT (ANG N-1498/+18) co-transfected with different concentrations of pBC-β_1AR in OK cells.* Results are expressed as the mean ± SD as control (without addition of isoproterenol or dexamethasone, 100%). Each point represents the mean ± SD of a minimum of three determinations (*$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.005$) (-▲-, control; –○–, isoproterenol; -●-, dexamethasone; -■-, isoproterenol and dexamethasone). Similar results were obtained from an additional experiment.

Dose-response curve of isoproterenol on the expression of pOCAT (ANG N-1498/+18) fusion gene co-transfected with pBC-β_1AR in OK cells in the presence or absence of dexamethasone

Figure 4 shows that the addition of high concentrations of isoproterenol (10^{-5} M) alone stimulated the expression of the pOCAT (ANG N-1498/+18) when co-transfected with 2 μg of pBC-β_1AR ($P \leq 0.05$). Smaller concentrations (less than 10^{-5} M) had no significant effect on the expression of the pOCAT (ANG N-1498/+18). In the presence of dexamethasone (10^{-6} M), the effect of isoproterenol (10^{-5} M) on the expression of the pOCAT (ANG N-1498/+18) was synergistically enhanced when compared to dexamethasone alone ($P \leq 0.05$) or isoproterenol alone ($P \leq 0.01$).

Synergistic effect of isoproterenol and dexamethasone on the expression of pOCAT (ANG N-1498/+18) co-transfected with pBC-β_1AR or pBC-β_2AR in OK cells

Figure 5 shows the synergistic effect of dexamethasone (10^{-6} M) and isoproterenol (10^{-5} M) on the expression of pOCAT (ANG N-1498/+18) when co-transfected with different DNA concentrations of pBC-β_1AR. It appears that the maximal synergistic effect of dexamethasone and isoproterenol was found at 2 μg pBC-β_1AR and was approximately 8-fold the control values (that is, without addition of dexamethasone or isoproterenol; $P \leq 0.005$). The combined effects of dexamethasone and isoproterenol were also significantly higher than the effect of dexamethasone or isoproterenol alone ($P \leq 0.005$).

On the other hand, it would appear that the stimulatory effect of a combination of dexamethasone and isoproterenol on the expression of pOCAT (ANG N-1498/+18) when co-transfected with pBC-β_2AR (1 to 5 μg DNA) was not significantly different from dexamethasone alone (Fig. 6), suggesting a lack of synergistic effect between dexamethasone and the β_2-adrenoceptor on the expression of pOCAT (ANG N-1498/+18) in OK cells.

These studies indicate that synergism is apparent between

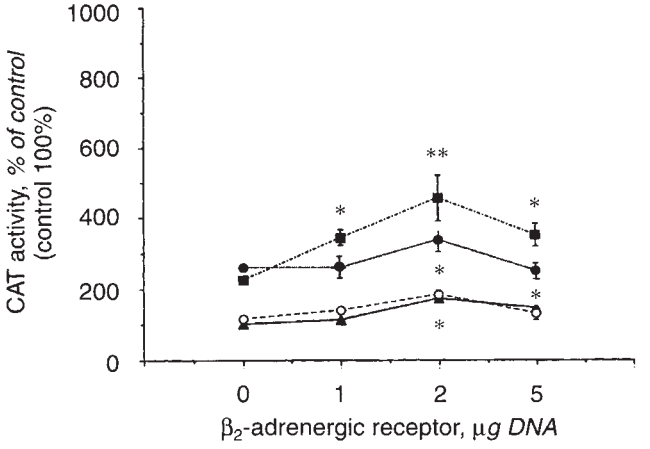

Fig. 6. *Effect of isoproterenol (10^{-5} M) or dexamethasone (10^{-6} M) or a combination of both on the expression of pOCAT (ANG N-1498/+18) co-transfected with different concentrations of pBC-β_2AR in OK cells.* Results are expressed as the mean ± SD as control (without addition of isoproterenol or dexamethasone, 100%). Each point represents the mean ± SD of a minimum of three determinations (*$P \leq 0.05$; **$P \leq 0.01$ and ***$P \leq 0.005$) (-▲- control; –○–, isoproterenol; -●-, dexamethasone; -■-, isoproterenol and dexamethasone). Similar results were obtained from an additional experiment.

dexamethasone and the β_1-adrenoceptor on the expression of pOCAT (ANG N-1498/+18), but not with the β_2-adrenoceptor present in OK cells.

Effect of RU 486 (an antagonist of glucocorticoid) and Rp-cAMP (an inhibitor of cAMP-dependent protein kinase A I and II) on the expression of pOCAT (ANG N-1498/+18) co-transfected with pBC-β_1AR in OK cells

Figure 7 illustrates that the overnight pre-incubation of OK cells which have been transfected with 10 μg pOCAT (ANG

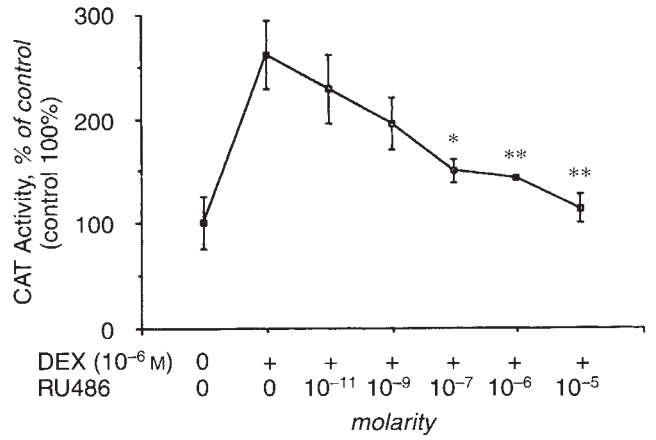

Fig. 7. *Inhibitory effect of RU 486 on the expression of pOCAT (ANG N-1498/+18) co-transfected with pBC-β_1AR in OK cells stimulated by dexamethasone.* Cells after DNA-transfection were pre-incubated with various concentrations of RU 486 for 16 hours and then further incubated for 24 hours in the presence of dexamethasone (10^{-6} M). Control cells (without the addition of RU 486 and dexamethasone) were given a relative value of 100%. Each point represents the mean ± SD of a minimum three determinations. The inhibitory effect of RU 486 was compared to cells which were incubated with 10^{-6} M dexamethasone but in the absence of RU 486 (*$P \leq 0.05$; **$P \leq 0.01$ and ***$P \leq 0.005$). Pre-incubation with RU 486 at 10^{-5} M consistently inhibited the stimulatory effect of dexamethasone (10^{-6} M) in another experiment.

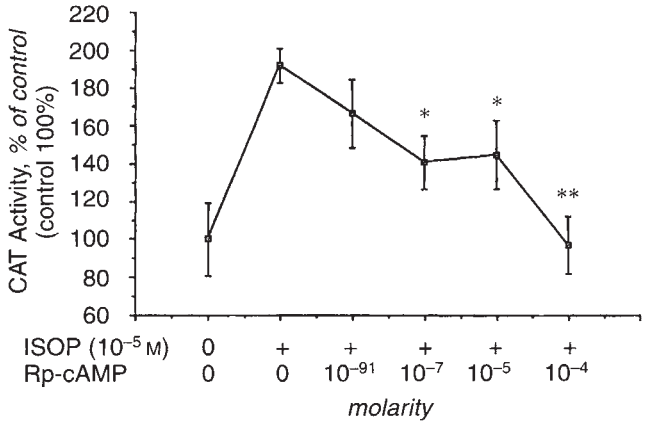

Fig. 8. *Inhibitory effect of (R)-p-adenosine 3′5′-cyclic monophospho-orthioate (Rp-cAMP) on the expression of pOCAT (ANG N-1498/+18) co-transfected with pBC-β_1AR in OK cells stimulated by isoproterenol.* Cells were incubated for up to 24 hours in presence of isoproterenol (10^{-5} M) with or without Rp-cAMP (10^{-10} to 10^{-4} M). Control cells (without addition of isoproterenol and Rp-cAMP) were given a relative value of 100%. The inhibitory effect of Rp-cAMP was compared to cells which were incubated with 10^{-5} M isoproterenol but in the absence of of Rp-cAMP. Each point represents the mean ± SD of a minimum of three determinations (*$P \leq 0.05$; **$P \leq 0.01$ and ***$P \leq 0.005$). Rp-cAMP at 10^{-4} M consistently inhibited the stimulatory effect of isoproterenol in another experiment.

N-1498/+18) and 2 μg pBC-β_1AR in the presence of different concentrations of RU 486 (10^{-11} to 10^{-5} M) blocked the stimulatory effect of dexamethasone (10^{-6} M) in a dose-dependent manner. The maximal and half-maximal inhibitory effects of RU 486 were found with 10^{-5} M ($P \leq 0.01$) and 10^{-7} M ($P \leq 0.05$), respectively.

The addition of Rp-cAMP (10^{-10} to 10^{-4} M) also blocked the expression of pOCAT (ANG N-1498/+18) when co-transfected with pBC-β_1AR in OK cells (Fig. 8). The maximal and half-maximal effective inhibitory effects of Rp-cAMP were found with 10^{-4} M ($P \leq 0.01$) and 10^{-7} M ($P \leq 0.05$), respectively.

Figure 9 shows the effect of RU 486 (10^{-5} M) or Rp-cAMP (10^{-4} M), or a combination of both, on the expression of pOCAT (ANG N-1498/+18) when co-transfected with pBC-β_1AR and stimulated by dexamethasone (10^{-6} M) and isoproterenol (10^{-5} M). The addition of Rp-cAMP (10^{-4} M) alone partially but significantly ($P \leq 0.05$) inhibited the synergistic effect of dexamethasone and isoproterenol on the expression of pOCAT (ANG N-1498/+18). The addition of RU 486 (10^{-5} M) alone or combined with Rp-cAMP (10^{-4} M) completely blocked the synergistic effect of dexamethasone and isoproterenol on the expression of pOCAT (ANG N-1498/+18; $P \leq 0.01$).

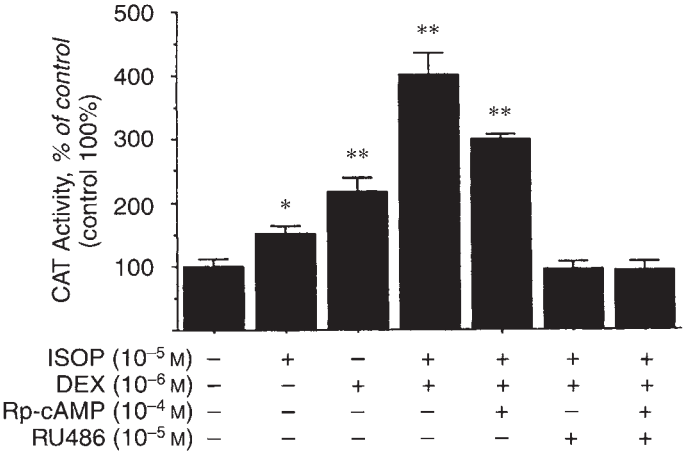

Fig. 9. *Effect of RU 486 (10^{-5} M) or Rp-cAMP (10^{-4} M) or a combination of both on the expression of pOCAT (ANG N-1498/+18) co-transfected with pBC-β_1AR in OK cells stimulated by isoproterenol (10^{-5} M) and dexamethasone (10^{-6} M).* Control cells (without addition of isoproterenol and dexamethasone) were given a relative value of 100%. Results are expressed as the mean ± SD of a minimum of three determinations (*$P \leq 0.05$; **$P \leq 0.01$ and ***$P \leq 0.005$). Similar results were obtained from three additional experiments.

Identification of putative cAMP-responsive element (CRE) and glucocorticoid-responsive element (GRE) in rat angiotensinogen gene

Figure 10 shows the effect of RU 486 (10^{-5} M) or Rp-cAMP (10^{-4} M), or a combination of both, on the expression of pTKCAT (ANG N-806/-465) in OK cells when co-transfected with pBC-β_1AR and stimulated by dexamethasone (10^{-6} M) and isoproterenol (10^{-5} M). Dexamethasone or isoproterenol alone stimulated the expression of pTKCAT (ANG N-806/-465) by 1.7-fold ($P \leq 0.05$) or 2.0-fold ($P \leq 0.01$), respectively. The addition of both dexamethasone and isoproterenol stimulated the expression of pTKCAT (ANG N-806/-465) by greater than 3.0-fold ($P \leq 0.01$). The addition of Rp-cAMP (10^{-4} M) alone partially but significantly ($P \leq 0.05$) inhibited the combined effect of dexamethasone and isoproterenol on the expression of pTKCAT (ANG N-806/-465). The addition of RU 486 (10^{-5} M) alone or combined with

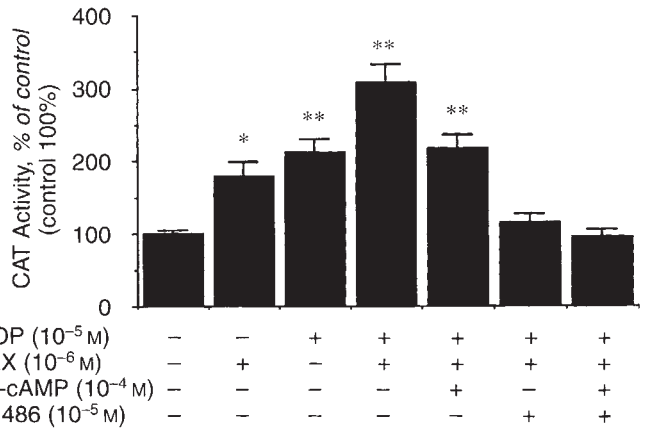

Fig. 10. *Effect of RU 486 (10^{-5} M) or Rp-cAMP (10^{-4} M) or a combination of both on the expression of pTKCAT (ANG N-806/-465) co-transfected with pBC-β_1AR in OK cells stimulated by isoproterenol (10^{-5} M) and dexamethasone (10^{-6} M).* Control cells (without addition of isoproterenol and dexamethasone) were given a relative value of 100%. Results are expressed as the mean ± SD of a minimum of three determinations (*$P \leq 0.05$; **$P \leq 0.01$ and ***$P \leq 0.005$). Similar results were obtained from another experiment.

Rp-cAMP (10^{-4} M) completely blocked the synergistic effect of dexamethasone and isoproterenol on the expression of pTKCAT (ANG N-806/-465; $P \leq 0.01$). These data indicate that the DNA fragment (ANG N-806 to N-465) contains both the cAMP-responsive element (CRE) and glucocorticoid-responsive elements (GREs) which are responsive to the addition of isoproterenol and dexamethasone.

Discussion

Recent studies by Nakamura and Johns [13] demonstrated that low levels of renal nerve stimulation decrease sodium excretion and increase the level of ANG mRNA in the rat kidney *in vivo*. The administration of the β_1-adrenoceptor antagonist, atenolol, blocked the effect of renal stimulation. This observation is supported by our previous studies [12] that isoproterenol directly stimulates the expression of the ANG gene in a dose-dependent manner in OK 27 cells, an OK cell line, into which has been stably integrated a fusion gene, pOGH (ANG N-1498/+18) containing the 5′-flanking regulatory sequence of the rat ANG gene fused with a human growth hormone (hGH) gene as reporter. The stimulatory effect of isoproterenol is blocked by the presence of propranolol, Rp-cAMP and atenolol. The addition of ICI 118,551 (an inhibitor of the β_2-adrenergic receptor), however, was 10,000-fold less effective in inhibiting the effect of isoproterenol as compared to atenolol. These studies suggest that the effect of isoproterenol is probably mediated via the β_1-adrenoceptor and not via the β_2-adrenoceptor. On the other hand, the lack of effectiveness of ICI 118,551 in inhibiting the stimulatory effect of isoproterenol in OK 27 cells would suggest that the β_2-adrenoceptor may be absent in OK 27 cells.

Our present studies showed that the co-transfection of the plasmid containing the cDNA for the β_1-adrenoceptor (pBC-β_1AR) or the β_2-adrenergic receptor (pBC-β_2AR) stimulated the basal expression of pOCAT (ANG N-1498/+18; Fig. 1), suggesting that these receptors may directly couple to the adenylyl cyclase system in OK cells. Indeed, studies have shown that these β_1- and β_2-adrenoceptor expression vectors are positively coupled to adenylyl cyclase when transfected into Chinese hamster fibroblasts [15–17].

It is surprising that the addition of isoproterenol (10^{-5} M) stimulated the expression of the pOCAT (ANG N-1498/+18) when co-transfected with pBC-β_1AR but not when co-transfected with either pBC-β_2AR or an empty vector, pBC-BC12 (Figs. 2, 5 and 6). These studies confirm our previous studies [12] that isoproterenol stimulates the expression of the pOGH (ANG N-1498/+18) in OK 27 cells via the β_1-adrenergic receptors. At present we do not understand why isoproterenol fails to enhance the effect of β_2-adrenoceptor in OK cells. One likely explanation is that there is a different degree (or rate) of desensitization (or sequestration) for the β_1- and β_2-adrenoceptors when exposed to high levels of agonists. Indeed, this possibility is supported by the studies of Suzuki et al [17] that the β_2-adrenoceptor is subjected to sequestration by its own agonist much faster than the β_1-adrenoceptor. That is, the β_2-adrenoceptor is subjected to a more rapid down-regulation than the β_1-adrenoceptor when exposed to isoproterenol [17–20]. Nevertheless, more experiments are warranted to clarify these possibilities in OK cells.

The addition of dexamethasone directly stimulated the expression of pOCAT (ANG N-1498/+18) when co-transfected with pBC-β_1AR in a dose-dependent manner (Fig. 3). The addition of both dexamethasone and isoproterenol acted synergistically to stimulate the expression of the pOCAT (ANG N-1498/+18) when co-transfected with pBC-β_1AR, but not with pBC-β_2AR (Figs. 4, 5 and 6). These data confirm our previous studies that dexamethasone directly stimulates the expression of pOCAT (ANG N-1498/+18) [11] and acts synergistically with 8-Br-cAMP to stimulate the ANG gene expression [14]. These data also support the presence of endogenous glucocorticoid receptor in OK cells as demonstrated by Vrtovsnick et al [21].

RU 486 is an antagonist of glucocorticoid and competes with glucocorticoid for binding to the glucocorticoid receptors [22]. Our present studies showed that pre-incubation of OK cells which have been transfected with pOCAT (ANG N-1498/+18) and pBC-β_1AR with RU 486 blocked the stimulatory effect of dexamethasone in a dose-dependent manner (Fig. 7). These studies demonstrate that the effect of dexamethasone on the expression of the ANG gene is mediated via the glucocorticoid receptor complex. Similarly, the addition of Rp-cAMP blocked the stimulatory effect of β_1-adrenoceptor on the expression of pOCAT (ANG N-1498/+18) in OK cells in a dose-dependent manner (Fig. 8). These studies indicate that the effect of β_1-adrenoceptor on the expression of the ANG gene is mediated via the cAMP-dependent protein kinase A pathway.

Interestingly, the addition of Rp-cAMP alone only partially blocked the stimulatory effect of dexamethasone and isoproterenol on the expression of pOCAT (ANG N-1498/+18) when co-transfected with pBC-β_1AR (Fig. 9), whereas the addition of RU 486 completely blocked the synergistic effect of dexamethasone and isoproterenol. The combined addition of RU 486 and Rp-cAMP also completely blocked the expression of pOCAT (ANG N-1498/+18) stimulated by dexamethasone and isoproterenol. These studies further confirm the apparent synergistic effect of dexamethasone and isoproterenol on the expression of the ANG gene.

Our transient gene transfection experiments showed that the addition of isoproterenol or dexamethasone alone stimulated the

expression of pTKCAT (ANG N-806/-465) when co-transfected with pBC-β_1AR (Fig. 10). The combined addition of isoproterenol and dexamethasone acted synergistically to stimulate the expression of the pTKCAT (ANG N-806/-465; Fig. 10). Our studies provide evidence that the putative CRE motif is probably located in the ANG N-795 to N-788 (TGACGTAC) region, whereas the GRE motifs are located in the ANG N-585 to N-579 (CAG AAC A) (GRE I) and N-478 to N-472 (CAG AAC A) (GRE II) regions [23]. Nevertheless, more experiments, such as transfection of fusion genes containing isolated CRE or GRE I or GRE II and site mutagenesis are necessary to demonstrate the exact motif of the CRE and GREs and experiments are underway in our laboratory to establish this location.

At present, the exact molecular mechanism(s) for the synergistic effect of isoproterenol (that is, via the β_1-adrenoceptor) and dexamethasone on the expression of the ANG gene in OK cells remains to be established. One possible explanation may be that the addition of isoproterenol stimulates the synthesis of intracellular cAMP in OK cells (which we have demonstrated previously [12]). The intracellular cAMP then binds to the regulatory subunit of cAMP-dependent protein kinase A (PKA), which subsequently releases the catalytic subunit of PKA. The active catalytic subunit of PKA translocates into the nucleus and phosphorylates both the cAMP-responsive element binding protein (CREB) or CREB-like proteins. The phosphorylated CREB or CREB-like protein(s) then interacts with the putative cAMP-responsive element (CRE) (that is, ANG N-795 to N-788, TGACGTAC) in the 5'-flanking region of the rat ANG gene [23], as well as an interaction with the phosphorylated glucocorticoid-receptor complexes (GRC) which are bound to the glucocorticoid responsive elements (GRE) (that is, ANG N-478 to N-472 and N-585 to N-579) [23] after stimulation by dexamethasone. Subsequently, the stabilized glucocorticoid receptor complex and phosphorylated CREB or CREB-like protein(s) will enhance the expression of the ANG gene. Indeed, this possibility is supported by studies of Imai et al [24], who showed that CREB can interact directly with the glucocorticoid receptor to stimulate the expression of the phosphoenolpyruvate carboxy kinase gene. Moreover, our present studies demonstrate that the addition of both isoproterenol and dexamethasone synergistically stimulated the expression of the pTKCAT (ANG N-806/-465; Fig. 10). Nevertheless, further experiments are warranted to confirm these possibilities with the ANG gene.

In summary, our studies demonstrate that isoproterenol and dexamethasone act synergistically to stimulate the expression of the ANG gene via the β_1-adrenergic receptor in OK cells. The addition of RU 486 and Rp-cAMP inhibited the synergistic effect of dexamethasone and isoproterenol. Thus, these studies raise the possibility that high cortisol levels in the plasma of patients with hypercortisolism may act synergistically with intracellular cAMP in the proximal tubular cells (via the activation of renal nerves under stress) to enhance the expression of the renal ANG gene. The local formation of renal Ang II might then modulate the physiology of the renal proximal tubular cells, including sodium and fluid reabsorption [25–30]. Hence, the local intra-renal renin angiotensin system may play an important role in the development of hypertension.

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References

1. Dzau VJ, Ingelfinger JR: Molecular biology and pathophysiology of the intrarenal renin-angiotensin system. *J Hypertens* 7(Suppl 7):53–58, 1989
2. Johnston CI, Fabris B, Jandeleit K: Intrarenal renin-angiotensin system in renal physiology and pathophysiology. *Kidney Int* 44(Suppl 42):559–563, 1993
3. Seikaly MG, Arant BS, Seney RD, Rutlege L, Green L: Endogenous angiotensin concentrations in specific intrarenal fluid compartments of the rat. *J Clin Invest* 86:1352–1357, 1990
4. Braam B, Mitchell KD, Fox J, Navar LG: Proximal tubular secretion of angiotensin II in rats. *Am J Physiol* 264:F891–F898, 1993
5. Ingelfinger JR, Zuo WM, Fon EA, Ellison KE, Dzau DJ: *In situ* hybridization evidence for angiotensinogen mRNA in the rat proximal tubule. A hypothesis for the intrarenal renin angiotensin system. *J Clin Invest* 85:417–423, 1990
6. Terada TK, Tomita K, Nonoguch H, Marumo F: PCR localization of angiotensin II receptor and angiotensinogen mRNA in rat kidney. *Kidney Int* 43:1251–1259, 1993
7. Wolf G, Neilson EG: Angiotensin II as a hypertrophogenic cytokine for proximal tubular cells. *Kidney Int* 43(Suppl 39):S100–S107, 1992
8. Tang SS, Jung F, Diamant D, Brown D, Bachinsky D, Hellman P, Ingelfinger JR: Temperature-sensitive SV 40 immortalized rat proximal tubule cell line has functional renin-angiotensin system. *Am J Physiol* 268:F435–F436, 1995
9. Chan JSD, Chan AHH, Nie Z-R, Sikstrom R, Lachance S, Hashimoto S, Carrière S: Thyroid hormone, L-T_3, stimulates the expression of the angiotensinogen gene in cultured opossum kidney (OK) cells. *J Am Soc Nephrol* 2:1360–1367, 1992
10. Ingelfinger JR, Boubounes B, Jun FF, Tang SS: High glucose downregulates expression of renin angiotensin system (RAS) in opossum kidney cells. (abstract) *Pediatr Nephrol* 29:344A, 1991
11. Chan JSD, Ming M, Nie Z-R, Sikstrom R, Lachance S, Carrière S: Hormonal regulation of expression of the angiotensinogen gene cultured in opossum kidney (OK) proximal tubular cells. *J Am Soc Nephrol* 2:1516–1522, 1992
12. Wang TT, Chen M, Lachance S, Delalandre A, Carrière S, Chan JSD: Isoproterenol and 8-Bromo-cyclic adenosine monophosphate stimulate the expression of the angiotensinogen gene in opossum kidney cells. *Kidney Int* 46:703–710, 1994
13. Nakamura A, Johns EJ: Effect of renal nerves on expression of renin and angiotensinogen genes in rat kidney. *Am J Physiol* 266:E230–E241, 1994
14. Ming M, Wang TT, Lachance S, Delalandre A, Carrière S, Chan JSD: Expression of the angiotensinogen gene is synergistically stimulated by 8-Br-cAMP and DEX in opossum kidney cells. *Am J Physiol* 268:R105–R111, 1995
15. Frielle T, Collin S, Daniel KW, Caron MG, Lefkowitz RJ, Kobilka BK: Cloning of the cDNA for the human β_1-adrenergic receptor. *Proc Natl Acad Sci USA* 84:7920–7924, 1987
16. Bouvier M, Hnatowich M, Collins S, Kobilka BK, Deblash A, Lefkowitz RJ, Caron MG: Expression of a human cDNA encoding the β_2-adrenergic receptor in Chinese hamster fibroblasts (CHW): Functionality and regulation of the expressed receptors. *Mol Pharmacol* 33:133–139, 1988
17. Suzuki T, Nguyen CT, Nantel F, Bonin H, Valiquette M, Frielle T, Bouvier M: Distinct regulation of β_1- and β_2-adrenergic receptors in Chinese hamster fibroblasts. *Mol Pharmacol* 41:542–548, 1992
18. Landsberg CR, Jastorff B: *In vitro* phosphorylation of microtubule-associated protein 2: Differential effects of cyclic AMP analogues. *J Neurochem* 45:1212–1222, 1985

19. KOYAMA H, GOODPASTURE C, MILLER MM, TEPLITZ RL, RIGGS AD: Establishment and characterization of a cell line from the American opossum (*Didelphys virginia*). *In Vitro* 14:239–246, 1978
20. POLLOCK AS, WARNOCK DG, STREWLER GJ: Parathyroid hormone inhibition of Na^+-H^+ antiporter activity in a cultured renal cell line. *Am J Physiol* 250:F217–F225, 1986
21. VRTOVSNIK F, JOURDAIN M, CHERQUI G, LEFEBVRE J, FRIEDLANDER G: Gluco-corticoid inhibition of Na-Pi cotransport in renal epithelial cells is mediated by protein kinase C. *J Biol Chem* 269:8872–8877, 1994
22. YANG JC, SERRES C, PHILIBERT D, ROBEL P, BEAULIEU EE, JOUANNET P: Progesterone and RU 486: Opposing effects on human sperm. *Proc Nat Acad Sci USA* 91:529–533, 1994
23. CHAN JSD, CHAN AHH, JIANG Q, NIE Z-R, LACHANCE S, CARRIÈRE S: Molecular cloning and expression of the rat angiotensinogen gene. *Pediatr Nephrol* 4:429–435, 1990
24. IMAI E, MINER JN, MITCHELL JA, YAMAMOTA KR, GRANNER KD: Glucocorticoid receptor-cAMP responsive element binding protein interaction and the response of the phosphoenolpyruvate carboxy kinase gene to glucocorticoids. *J Biol Chem* 268:5353–5356, 1993
25. STEVEN K: Effect of peritubular infusion of angiotensin II on rat proximal nephron function. *Kidney Int* 6:73–80, 1974
26. HALL JE, GUYTON AC, TRIPPODO NC, LOHMEIER TE, MCCREA RE, COWLEY AWL JR: Intrarenal control of electrolyte excretion by angiotensin II. *Am J Physiol* 232:F538–F544, 1977
27. HARRIS PJ, YOUNG YA: Dose-dependent stimulation and inhibition of proximal tubular sodium reabsorption by angiotensin II in rat kidney. *Pflügers Arch* 367:1295–1297, 1977
28. SCHUSTER VL, KOKKO JP, JACOBSON HR: Angiotensin II directly stimulates sodium reabsorption by angiotensin II in the kidney. *J Clin Invest* 73:507–515, 1984
29. LIU FY, COGAN MG: Angiotensin II stimulates early proximal bicarbonate absorption in the rat by decreasing cyclic adenosine monophosphate. *J Clin Invest* 84:83–91, 1989
30. WANG T, CHAN YL: Mechanism of angiotensin II action on proximal tubular transport. *J Pharmacol Exp Ther* 252:689–695, 1990